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Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase

ERIC N. JOHNSON*, LAWRENCE F. BRASS†‡, AND COLIN D. FUNK‡§

*Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232; and †Departments of Medicine and Pathology and ‡Center for Experimental Therapeutics, University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT **Arachidonic acid metabolism is one of several mechanisms culminating in the production of an agonist for platelet activation and recruitment. Although the proaggregatory role of thromboxane A_2, a product of the aspirin-inhibitable cyclooxygenase, is well established, relatively little is known regarding the biological importance of arachidonic acid metabolism via the 12-lipoxygenase (P-12LO) pathway to 12-hydro(pero)xyeicosatetraenoic acid. We observed that platelets obtained from mice in which the P-12LO gene has been disrupted by gene targeting (P-12LO$^{-/-}$) exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in *ex vivo* assays and increased mortality in an ADP-induced mouse model of thromboembolism. The hyperresponsiveness to ADP is independent of dense granule release, cyclooxygenase-derived eicosanoid synthesis, and protein kinase C activity. The addition of 12-hydroxyeicosatetraenoic acid to P-12LO$^{-/-}$ platelet-rich plasma rescues the hyperresponsive phenotype resulting in a diminished ADP-induced aggregation profile. The enhanced ADP sensitivity of P-12LO$^{-/-}$ mice appears to reveal a mechanism by which a product of the P-12LO pathway suppresses platelet activation by ADP.**

The platelet-type 12-lipoxygenase (P-12LO) is a member of the lipoxygenase family expressed in platelets, megakaryocytes, epidermal cells, and some tumor cell lines (1–4). P-12LO catalyzes the stereospecific oxygenation of arachidonic acid to form 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (5, 6), which is reduced by a glutathione peroxidase or other mechanisms to 12-hydroxyeicosatetraenoic acid (12-HETE) (7, 8). Although a leukocyte-type 12-lipoxygenase and an epidermal-type 12-lipoxygenase exist in mice that are also capable of 12-HETE biosynthesis, the distribution of these enzymes is distinct from that of P-12LO (1–4, 9, 10).

In the activated platelet, arachidonic acid is a common substrate for the cyclooxygenase/thromboxane synthase pathway and the P-12LO pathway. Although the proaggregatory role for thromboxane A_2 in platelet function is clearly understood, the importance of P-12LO in platelet biology is not evident (11). Thromboxane formation is rapid and quickly reaches a plateau in the activated platelet whereas 12-H(P)ETE formation is slower and continues to accumulate over a longer time course (12). 12-Lipoxygenase metabolites can undergo further transformations in cell–cell interactions (e.g., platelet–neutrophil interactions) leading to the formation of lipoxins and 12,20-di-HETE, which may regulate cell activity (13, 14). Thus, we sought to explore potential roles for the P-12LO pathway by using gene targeting techniques to create P-12LO-deficient mice. Our studies have revealed a selective modulatory role for P-12LO in the ADP-induced pathway of platelet aggregation in mice.

EXPERIMENTAL PROCEDURES

P-12LO Gene Targeting. A 5.9-kb *Xba*I fragment encoding exons 9–11 of the mouse P-12LO gene (9) was cloned into the unique *Xba*I site of the vector pPNT (15), followed by a 2.7-kb *Sca*I–*Xho*I blunted fragment encoding exons 3–6 into a blunted *Xho*I site upstream of the neomycin-resistance cassette. Culture and electroporation of D3H-ES cells were performed as described (16). Confirmation of homologous recombination and germ-line transmission were performed by Southern blot analysis using initially a probe external to the targeting vector and subsequently a probe covering exons 1 and 2, as described (16). Mating of P-12LO$^{+/-}$ mice yielded the expected 1:4 ratio of P-12LO$^{-/-}$ offspring. All mice used in these experiments were C57BL/6× 129 Sv strain. Control animals were age/sex-matched mice of the same genetic hybrid background bred in our colony or purchased from The Jackson Laboratory.

Platelet Isolation. Male mice (20–25 g) anesthetized with volatile isofluorane were used for cardiac puncture. The heart was exposed and a 1-ml syringe with a 23-gauge needle containing 100 μl of 3.8% sodium citrate was used to obtain about 1 ml of blood. The blood was centrifuged at 100 × *g* for 10 min. The platelet-rich plasma (PRP) was removed and approximately 200 μl of Hepes/Tyrode's (H/T) buffer (129 mM NaCl/8.9 mM $NaHCO_3$/2.8 mM KCl/0.8 mM KH_2PO_4/5.6 mM dextrose/10 mM Hepes/0.8 mM $MgCl_2$, pH 7.4) was added back to the blood. The sample was mixed by inversion and centrifuged at 100 × *g* for 10 min. A second aliquot of PRP was removed and combined with the first. Platelets were counted in a Coulter counter and the platelet number was adjusted to 3.5×10^8 platelets per ml with H/T buffer.

Detection of P-12LO Protein. Protein (15 μg) from platelet sonicates was separated by SDS/PAGE on 10% gels, blotted to nitrocellulose, and probed with an anti-six-histidine-tagged-human P-12LO antibody (17).

Measurement of P-12LO Activity by HPLC. Protein (200 μg) from platelet sonicates was incubated with 100 μM arachidonic acid at 37°C for 15 min. Samples were extracted and analyzed by reverse-phase HPLC (18) by using a solvent system of acetonitrile/methanol/water/acetic acid, 350:150:250:1 (vol/vol) at a flow rate of 1.5 ml/min. The effluent was monitored at 235 nm.

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Abbreviations: P-12LO, platelet-type 12-lipoxygenase; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; PKC, protein kinase C; PRP, platelet-rich plasma; 12-(*S*)-HPETE, 12-(*S*)-hydroperoxyeicosatetraenoic acid; COX-1, cyclooxygenase 1.

§To whom reprint requests should be addressed at: 805 Stellar-Chance Laboratories, Center for Experimental Therapeutics, 422 Curie Boulevard, University of Pennsylvania, Philadelphia, PA 19104. e-mail: colin@spirit.gcrc.upenn.edu.

Platelet Aggregation. Platelet aggregation was measured by using a lumi-aggregometer (Chrono-Log, Havertown, PA). Samples were incubated at 37°C and percent light transmission was measured compared with a platelet-poor plasma blank. Final volume of aggregation was 250 μl. Platelet stimulants and inhibitors are given in final concentrations. Comparison of the rate and extent of aggregation in wild-type and P-12LO$^{-/-}$ mice was evaluated by the Student's *t* test. Investigators were blinded as to the genotype of the mice during all aggregation experiments.

For indomethacin studies, vehicle (EtOH), or indomethacin (1 μM) (Sigma) was added and samples incubated for 5 min at 37°C. Aggregation was stimulated by either 100 μM arachidonic acid (Calbiochem) or 8 μM ADP (Chrono-Log), final concentration.

For protein kinase C (PKC) studies, the PKC-specific inhibitor GF109203X (5 μM; Calbiochem) (19) or vehicle (1 μl of dimethyl sulfoxide) was added to each platelet sample. After a 5-min incubation, phorbol 12-myristate 13-acetate or ADP (100 nM and 8 μM) was used to induce aggregation.

For detection of ATP release by luminescence, 10 μl of Chrono-lume (Chrono-Log) was added to 230 μl of PRP. Aggregation was stimulated by adding 10 μl of agonist to a final thrombin concentration of 1 unit/ml (Sigma) or 8 μM ADP (Chrono-Log). ATP (Chrono-Log; 600 pmol) was used as internal standard.

For rescue experiments, PRP from P-12LO$^{-/-}$ mice was supplemented with either 12-(*S*)-hydroperoxyeicosatetraenoic acid [12-(*S*)-HPETE] (1–200 μM; a gift from Alan R. Brash, Vanderbilt University, Nashville, TN), 12-(*S*)-HETE (1–200 μM; Biomol, Plymouth Meeting, PA), or vehicle (ethanol). Aggregation was stimulated with either 8 μM ADP or 10 μM U46619 (Calbiochem).

***In Vivo* Thrombosis Models.** Female mice were weighed and various concentrations of ADP dissolved in H/T were injected via the tail vein (20). At a dose of 0.035 mg of ADP/g (body weight), death occurred within 2 min, or mice were monitored for 15 min, before being recorded as survivors prior to sacrifice. Arachidonic acid (sodium salt) in PBS at a concentration of 30 or 100 mg/kg was injected via the tail vein in separate experiments. Investigators were blinded to the genotype of the animals during the experiment. Data were analyzed by the Fisher's exact statistical test.

RESULTS

P-12LO Gene Targeting. To define the role of P-12LO and its arachidonate metabolites 12(*S*)-hydro(pero)xyeicosatetraenoic acid *in vivo*, we disrupted the P-12LO gene (9) in mice via gene targeting (Fig. 1 *A* and *B*). Multiple lines of evidence confirm the successful disruption of P-12LO in these animals. A P-12LO antibody detected an ~75-kDa band in P-12LO$^{+/+}$ and P-12LO$^{+/-}$ platelet sonicates but not in sonicates from P-12LO$^{-/-}$ platelets (Fig. 1*C*). Platelets from P-12LO$^{-/-}$ mice failed to synthesize 12-HPETE, or its related metabolites, in contrast to wild-type animals. However, products derived from the cyclooxygenase pathway (thromboxane B_2 and 12-hydroxyheptadecatrienoic acid) were not detectably altered by the gene disruption in the presence of exogenous arachidonic acid either at high or tracer concentrations (Fig. 1*D*). In addition,

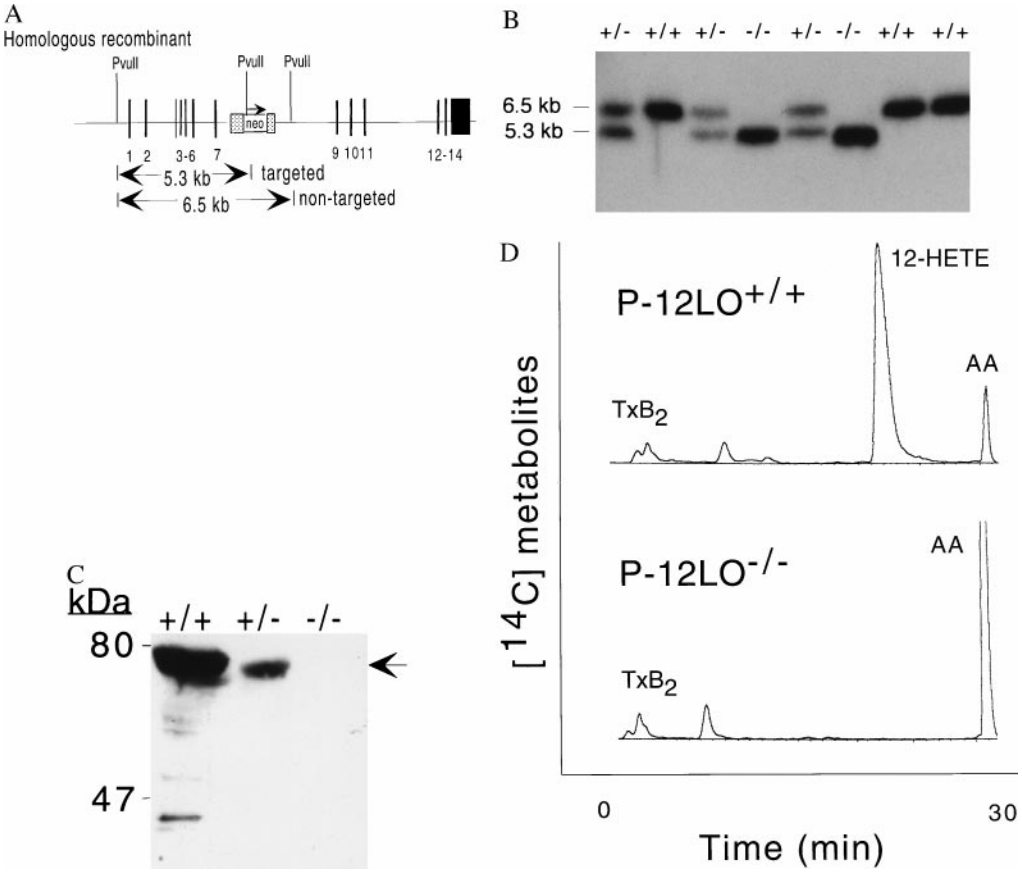

FIG. 1. Disruption of the P-12LO gene in mice. (*A*) The predicted homologous recombinant in which exon 8 was deleted and replaced with a neomycin cassette. An additional *Pvu*II restriction endonuclease site in the neomycin cassette was used to detect the presence of the mutant allele. (*B*) Southern blot in which mouse tail DNA was digested with *Pvu*II; hybridization with labeled probe encoding exons 1–2 was used to detect the presence of only the mutant allele (5.3 kb; P-12LO$^{-/-}$), only the wild-type allele (6.5 kb; P-12LO$^{+/+}$), or both (P-12LO$^{+/-}$) by autoradiography. (*C*) Western blot detection of P-12LO protein. Arrow shows the position of P-12LO immunoreactive protein. (*D*) HPLC chromatograms of products formed from incubation of platelets with [^{14}C]arachidonic acid (0.3 μCi, 100 μM; 1 Ci = 37 GBq).

the leukocyte-type 12-lipoxygenase (1), an isozyme expressed predominantly in mouse peritoneal macrophages (21) and the product of a separate but linked gene (9), is expressed normally in these mice as revealed by HPLC analysis of arachidonic acid metabolites synthesized by peritoneal macrophages (data not shown).

Platelet Studies Reveal an Increased Aggregation of P-12LO$^{-/-}$ Platelets in Response to ADP. P-12LO$^{-/-}$ mice are fertile and appear to exhibit no gross abnormalities up to 18 months of age, the longest time evaluated. P-12LO has been postulated by some investigators to play a role in megakaryocytic development and platelet maturation (22); however, the numbers of platelets and megakaryocytes in P-12LO$^{-/-}$ mice do not differ from those of P-12LO$^{+/+}$ mice (mean platelet number for male mice is 988, 912, and 988 $\times$ 10^3 platelets per μl for P-12LO$^{+/+}$, P-12LO$^{+/-}$, and P-12LO$^{-/-}$, respectively; n = 2–6). To examine the role of P-12LO in platelet function, adhesion to extracellular matrix proteins, platelet aggregation, and granule secretion were evaluated. Adhesion assays comparing P-12LO$^{-/-}$ and P-12LO$^{+/+}$ platelets revealed no difference in platelet adhesion to various extracellular matrix proteins including fibrinogen, collagen, and fibronectin (data not shown). P-12LO-deficient platelets stimulated by a range of concentrations of platelet agonists including thrombin, collagen, arachidonic acid, and U46619 (a thromboxane A_2 mimetic agent) exhibited percent aggregation responses comparable to those elicited by wild-type platelets (Fig. 2). However, activation of P-12LO$^{-/-}$ mouse platelets in response to multiple concentrations of ADP resulted in a statistically significant hyperresponsiveness manifested as an increase in slope and percent light transmittance over P-12LO$^{+/+}$ mouse platelets (Fig. 3). The aggregation was reversible up to 40 μM ADP concentrations in mice of both genotypes.

Because cyclooxygenase 1 (COX-1) and P-12LO share a common substrate (5, 6) (arachidonic acid), increased ADP-induced aggregation in P-12LO$^{-/-}$ mice might result from enhanced thromboxane A_2 production in platelets derived from these animals. P-12LO$^{+/+}$ and P-12LO$^{-/-}$ platelets were

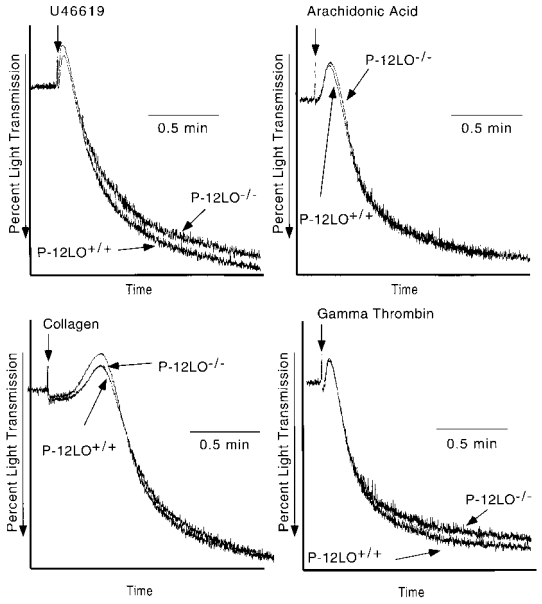

FIG. 2. Thrombin, collagen, U46619, and arachidonic acid-induced aggregation responses are largely unaffected by disruption of the P-12LO gene. Agonist was added to PRP as indicated by the arrow. Extent of aggregation of P-12LO$^{-/-}$ mouse platelets was not significantly different from wild-type control mouse platelets stimulated with γ thrombin (10–100 nM), collagen (2–40 μg/ml), U46619 (0.5–20 μM), or arachidonic acid (20–100 μM) (n = 4 to 6).

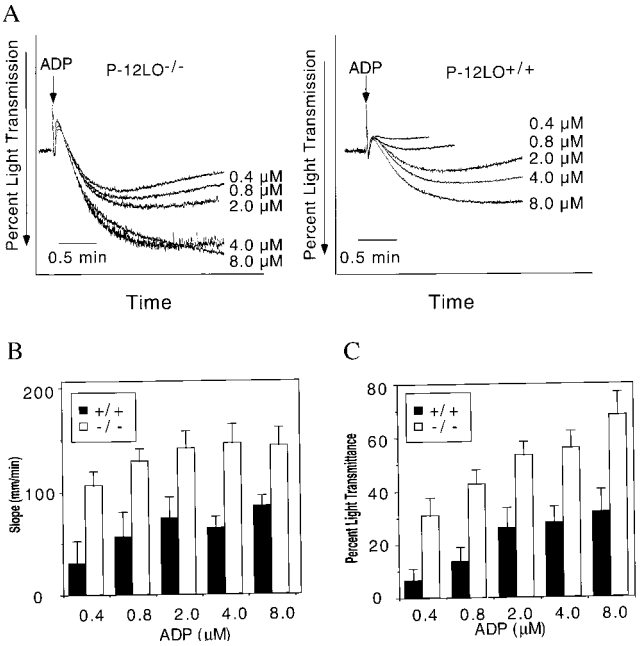

FIG. 3. ADP-stimulated aggregation is uniquely altered in P-12LO$^{-/-}$ mice. (*A*) Aggregation profiles of P-12LO$^{+/+}$ and P-12LO$^{-/-}$ platelets at the indicated doses of ADP. (*B* and *C*) Bar graphs depict the mean slope (*B*) and percent of platelet aggregation (*C*) of eight +/+ (solid bars) and eight −/− (open bars) mice. Error bars are the SEM. At each dose the difference is significant, as determined by Student's t test ($P < 0.05$).

treated with indomethacin to inhibit COX-1 activity, and hence thromboxane A_2 production, before aggregation stimulated by ADP. Treatment of platelets with indomethacin did not alter either the slope or the percent of ADP-induced aggregation of platelets derived from mice of either genotype (Fig. 4*A Lower*), demonstrating not only that the difference in ADP sensitivity between P-12LO$^{-/-}$ and P-12LO$^{+/+}$ platelets was not secondary to enhanced thromboxane A_2 synthesis in P-12LO$^{-/-}$ platelets but also that COX-1 (indomethacin-sensitive)-generated eicosanoids were not required for ADP-induced aggregation of mouse platelets, in contrast to expectations for human platelets (23).

The involvement of PKC in downstream signaling events evoked by ADP was explored. Preincubation of platelets with GF109203X, an inhibitor of PKC (19), completely blocked aggregation stimulated by phorbol 12-myristate 13-acetate in wild-type and P-12LO$^{-/-}$ platelets (Fig. 4*B*). However, this concentration of GF109203X failed to alter the rate or extent of platelet aggregation stimulated with ADP in P-12LO$^{-/-}$ or P-12LO$^{+/+}$ mice, providing evidence that PKC activation does not contribute to the ADP hypersensitivity of P-12LO$^{-/-}$ platelets. The rate of aggregation reversal does appear to increase in GF109203X-treated platelets from wild-type but not P-12LO$^{-/-}$ mice, implicating a role for PKC in stabilization of platelet aggregates that is either downstream of a P-12LO signaling pathway or masked by the increased aggregation of P-12LO$^{-/-}$ mouse platelets.

The release of the contents of platelet dense granules (including ADP and fibrinogen) is thought to play an important role in perpetuating the aggregation response (24). Liberated ATP, quantified via a luciferin–luciferase assay (25), was used as a measure of dense granule release (Fig. 4*C*). Although thrombin (1 unit/ml) induced release of dense granules in all mice, ADP (8 μM) failed to evoke dense granule release in mice of either genotype, indicating that hypersensitivity to ADP in P-12LO$^{-/-}$ platelets is not secondary to dense granule release.

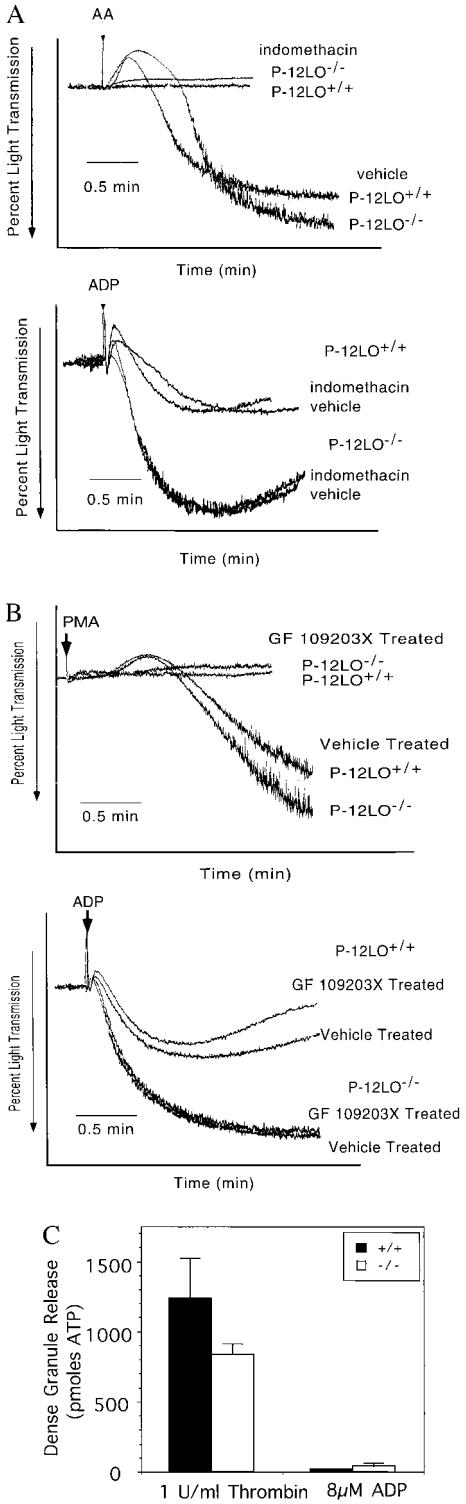

FIG. 4. Analysis of second messenger systems in ADP-induced platelet aggregation. The lack of role of COX-1-derived thromboxane synthesis (*A*) and PKC (*B*) in ADP-stimulated aggregation of P-12LO$^{+/+}$ and P-12LO$^{-/-}$ platelets (*Lower*). Platelets were incubated with either 1 μM indomethacin or 5 μM GF109203X before aggregation. Aggregation by arachidonic acid or phorbol 12-myristate 13-acetate was completely inhibited by incubation with indomethacin or GF109203X, respectively (*Upper*). Traces are representative of experiments performed with platelets from two mice. (*C*) Measurement of ATP release upon stimulation of P-12LO$^{+/+}$ (solid bars) and $^{-/-}$ (open bars) platelets with thrombin (1 unit/ml) and 8 μM ADP (n = 3).

To confirm that the lack of 12-H(P)ETE is responsible for the increased ADP-induced aggregation, 12-HPETE and 12-HETE were added to P-12LO$^{-/-}$ PRP before stimulation. The addition of 12-HPETE (10 μM final) resulted in a slight decrease in aggregation (<10%). A final concentration of 200 μM 12-HPETE attenuated ADP-induced aggregation up to 50%; however, aggregation in response to either U46619 or γ thrombin were completely inhibited at such concentrations. 12-(*S*)-HETE (1–200 μM, final concentration) was added to PRP from P-12LO$^{-/-}$ mice. A final concentration of 200 μM 12-(*S*)-HETE attenuated the ADP-induced aggregatory response from 82% to 63%, indicating that the difference in aggregation between P-12LO$^{-/-}$ and P-12LO$^{+/+}$ platelets is likely due to the lack of 12-HETE during the aggregation process (Fig. 5). These concentrations of 12-HETE did not alter U46619-induced platelet aggregation.

Thrombosis Models Confirm the Hyperactivation of P-12LO$^{-/-}$ Platelets *in Vivo*. To evaluate the *in vivo* consequences of a lack of P-12LO, we examined a model of ADP-induced thrombosis (20) in which a series of platelet-activating events culminates in death. Dose–response studies revealed that ADP at a dose of 0.035 mg/g (body weight) elicited 20% mortality in P-12LO$^{+/+}$ mice; however, this same dose evoked an 87.5% mortality rate in P-12LO$^{-/-}$ mice (Fig. 6), confirming the importance *in vivo* of P-12LO in attenuating sensitivity to ADP. In contrast, the use of arachidonic acid (30 and 100 mg/kg) to induce thrombosis resulted in the same incidence of mortality.

DISCUSSION

P-12LO-deficient mice have been generated by targeted gene disruption. Platelets from these mice lack the ability to synthesize 12-HETE from exogenous or endogenous arachidonic acid, whereas thromboxane synthesis appears to remain unchanged (Fig. 1). P-12LO-deficient mouse platelets exhibit hyperresponsiveness to ADP (Fig. 3) that is not secondary to thromboxane synthesis, PKC activity, or dense granule release (Fig. 4). Rescue experiments have shown that the addition of 12-(*S*)-HETE to P-12LO$^{-/-}$ PRP can attenuate the ADP-induced aggregatory response (Fig. 5). The high concentration of 12-HETE required to cause this effect may reflect extensive binding of the compound by plasma proteins. Similar concentrations of arachidonic acid were required to elicit aggregation of PRP. These data suggest a role for P-12LO as part of an inhibitory pathway unique to ADP-induced aggregation. In an *in vivo* model of thrombosis, P-12LO-deficient mice exhibit thromboembolytic death

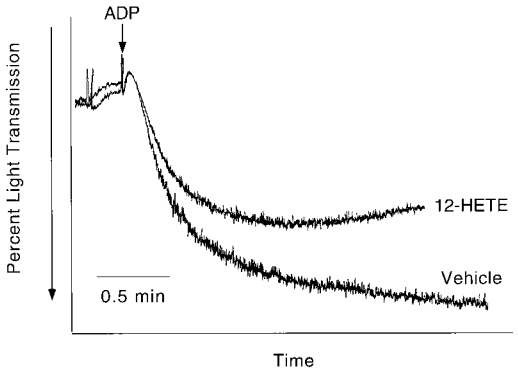

FIG. 5. Hyperaggregation in ADP-stimulated P-12LO$^{-/-}$ platelets can be attenuated by adding back 12-HETE. PRP was prepared as described above. Immediately after the addition of 12-HETE (200 μM, final concentration) or vehicle (EtOH), ADP (8 μM, final concentration) was added to induce aggregation. The curve shown is a representative tracing of a decrease in percent aggregation from 82.5% to 63.1%. A Student's t test of paired experiments yielded a two-tailed P value of 0.0032 (n = 12).

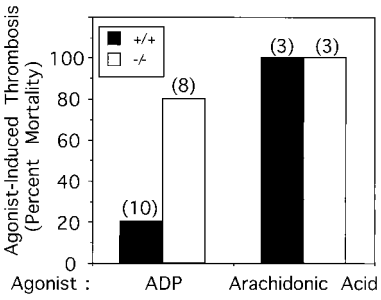

FIG. 6. P-12LO$^{-/-}$ mice are more sensitive to thrombosis elicited by intravenous ADP injection. Female mice were injected with ADP at 0.035 mg/g (body weight) via the tail vein. The percent mortality of P-12LO$^{+/+}$ (solid bars) and P-12LO$^{-/-}$ (open bars) mice is indicated. The number of mice is indicated in parentheses. ($P < 0.05$, as determined by Fisher's exact test). Differences in mortality with arachidonic acid-induced thrombosis were not observed at 30 mg/kg (data not shown) and 100 mg/kg.

at a dose of ADP that fails to elicit death in wild-type control mice, thus extending our *ex vivo* findings. Because ADP and adenosine [a potential product of metabolism of the thrombomodulatory ecto-ADPase (26)] are vasodilatory compounds, we cannot rule out absolutely a platelet-independent contributing factor for the differences in mortality observed with P-12LO deficiency.

12-Lipoxygenase products can undergo transcellular metabolism to lipoxins (13) and 12,20-di-HETE (14). Although not explored in this article, it should be interesting to see the consequence of loss of synthesis of these products in appropriate models of inflammation where platelet–neutrophil interactions are important. There has been some speculation that the P-12LO pathway is involved in megakaryocytopoiesis and platelet shedding, somewhat like the 15-lipoxygenase of rabbit reticulocytes believed to contribute to membrane degradation and maturation of the red cell (27). Because disruption of the P-12LO pathway did not alter the number of circulating platelets, its role in thrombopoiesis is not crucial. However, detailed studies of megakaryocytes isolated from P-12LO-deficient mice may reveal a more subtle role in megakaryocytic development or function.

Platelets from P-12LO-deficient mice exhibit essentially normal aggregatory responses to U46619, arachidonic acid, collagen, and γ thrombin (Fig. 2). These findings raise speculation regarding the physiological relevance of previous reports that conclude that P-12LO metabolites inhibit the binding of thromboxane A_2 receptor ligands (28), interfere with collagen and arachidonic acid-induced aggregation (29), and enhance thrombin-induced aggregation (30). These disparate results could be caused by interspecies variation or differences in methodology, because previous experiments involved the incubation of human platelets with an excess of 12-H(P)ETE. A diminished aggregatory response to U46619 or thrombin was observed upon treatment of human platelets with three lipoxygenase inhibitors (31). These compounds may have had actions in addition to the inhibition of P-12LO that would account for the discrepancy in results. Alternatively, divergent conclusions could be explained by differences in signaling in the P-12LO pathway of mice and humans. Species variations have been reported for epinephrine (ref. 32 and unpublished data) and the thrombin receptor activation peptide (33) that fail to stimulate mouse platelets despite their ability to induce aggregation of human platelets.

In conclusion, we have created an *in vivo* system that lacks P-12LO. A role for P-12LO in specifically attenuating ADP-induced platelet aggregation has been demonstrated. The revelation that such a pathway exists provides a potential mechanism of the underlying thrombohemorrhagic predisposition observed in described cases of P-12LO deficiency in primary thrombocythemia (34). A striking similarity of attenuated ADP-induced aggregation is seen in mice that lack the adenosine A_{2a} receptor (35), indicating that perhaps P-12LO is involved in a signaling pathway downstream of this receptor or an additional pathway that appears to be functionally redundant. Studies such as these offer clues to reveal the elusive pathway of ADP-stimulated platelet aggregation.

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